Spontaneous Release of Cytosolic Proteins from Posttranslational Substrates before their Transport into the Endoplasmic Reticulum

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Abstract. In posttranslational translocation in yeast, completed protein substrates are transported across the endoplasmic reticulum membrane through a translocation channel formed by the Sec complex. We have used photo-cross-linking to investigate interactions of cytosolic proteins with a substrate synthesized in a reticulocyte lysate system, before its posttranslational translocation through the channel in the yeast membrane. Upon termination of translation, the signal recognition particle (SRP) and the nascent polypeptide–associated complex (NAC) are released from the polypeptide chain, and the full-length substrate interacts with several different cytosolic proteins. At least two distinct complexes exist that contain among other proteins either 70-kD heat shock protein (Hsp70) or tailless complex polypeptide 1 (TCP1) ring complex/chaperonin containing TCP1 (TRiC/CCT), which keep the substrate competent for translocation. None of the cytosolic factors appear to interact specifically with the signal sequence. Dissociation of the cytosolic proteins from the substrate is accelerated to the same extent by the Sec complex and an unspecific GroEL trap, indicating that release occurs spontaneously without the Sec complex playing an active role. Once bound to the Sec complex, the substrate is stripped of all cytosolic proteins, allowing it to subsequently be transported through the membrane channel without the interference of cytosolic binding partners.

Key words: cytosolic chaperones • endoplasmic reticulum • posttranslational protein translocation • Sec complex • yeast

Introduction

It is generally believed that proteins are transported through a protein-conducting channel of the ER membrane in an unfolded conformation. In cotranslational translocation, an unfolded state is maintained simply by the fact that the nascent polypeptide chain is transported while being synthesized on a ribosome that is bound to the membrane channel. In contrast, for posttranslationally transported proteins there must be a mechanism that prevents their aggregation or premature folding into a stable structure before they are transported through the channel. Cytosolic chaperones are likely involved to keep a translocation substrate in an unfolded or loosely folded conformation before it enters the channel (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988; Caplan et al., 1992; Chirico, 1992). However, the identity of the chaperones and the mechanism by which they function remain largely unknown. In addition, once bound to the substrate, chaperones and other interacting cytosolic proteins would seem to pose a problem for the subsequent translocation step. How are they released to allow the polypeptide chain to slide through the channel? One possibility is that there is an active mechanism by which they are released when the substrate binds to or translocates through the channel. Alternatively, release may occur spontaneously in the cytosol, and the membrane channel may only capture free polypeptide chains.

Posttranslational translocation occurs both in yeast and in mammalian cells, although in the latter case only substrates smaller than 70 amino acids are translocated and the mechanism of transport is not well understood (Zimmermann et al., 1988). Studies in yeast have shown that the posttranslational translocation channel is formed from a seven-component membrane protein complex, the Sec complex (Panzner et al., 1995). The Sec complex binds the translocation substrate through its signal sequence. The latter intercalates into two transmembrane domains of the Sec61p subunit of the complex, resulting in the insertion of the polypeptide chain into the channel as a loop (Plath et al., 1998). The subsequent movement of the chain through the channel involves the function of lumenal Kar2p (also called BiP), a member of the 70-kD heat shock protein

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(Hsp70) family in the lumen of the ER (Vogel et al., 1990; Brodsky and Schekman, 1993). At least in the case of the translocation substrate prepro-α factor (ppαF, 165 amino acids), Kar2p can function as a molecular ratchet to move the polypeptide chain across the membrane (Matlack et al., 1999). It binds to the incoming polypeptide chain and prevents its backsliding through the channel.

Cytosolic chaperones implicated in posttranslational protein translocation include Hsp70 and its cofactor, the J protein Ydj1p (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988; Caplan et al., 1992; Becker et al., 1996). Experiments in *Saccharomyces cerevisiae* have shown that loss-of-function mutants in the corresponding genes affect the translocation of some proteins (Deshaies et al., 1988; Caplan et al., 1992; Becker et al., 1996). In addition, biochemical experiments have provided evidence that Hsp70 can associate with the substrate ppαF and can stimulate its posttranslational translocation in vitro (Chirico et al., 1988; Chirico, 1992). It is unknown whether chaperones other than the Hsp70–Ydj1p system interact with posttranslational translocation substrates. Significantly, however, cytosolic proteins are not necessary for the translocation process per se. The requirement for the binding and release of cytosolic factors can be bypassed by denaturing the substrate in urea, and the translocation process can be reconstituted with purified components in the absence of any cytosolic proteins (Chirico et al., 1988; Matlack et al., 1999). In addition, the Hsp70–Ydj1p system is required for translocation into both the ER and mitochondria. Thus, it is possible that cytosolic chaperones may only keep polypeptides in a loosely folded conformation and may have no specific interactions with either targeting signals or the translocation machinery in the membrane.

While it is conceivable that a posttranslational translocation substrate interacts with the same cytosolic chaperones as a polypeptide chain remaining in the cytosol, some differences may exist. For polypeptides lacking signal sequences, Hsp70 and Hsp40 (a mammalian cytosolic J protein) have been found to associate with ribosome-bound nascent chains (Frydman et al., 1994; Frydman and Hartl, 1996; Eggers et al., 1997; Pfund et al., 1998). In addition, the chaperonin-containing tailless complex polypeptide 1 (TCP1)[1] complex (TRiC/CCT; for review see Kim et al., 1994) has been reported to interact with ribosome-bound nascent chains (Frydman et al., 1994; Frydman and Hartl, 1996; McCallum et al., 2000), unlike the bacterial homologue GroEL, which only binds completed polypeptides (Netzer and Hartl, 1998). Ribosome-bound, but not full-length polypeptide chains also interact with the nascent polypeptide–associated complex (NAC) (Wiedmann et al., 1994). Although no systematic study on the interaction with cytosolic proteins has been performed for posttranslational translocation substrates, the situation may be quite different. During synthesis of the polypeptide chain, the signal sequence likely interacts with the signal recognition particle (SRP), although the interaction may be weaker than with the more hydrophobic signal sequences that direct substrates into the cotranslational translocation pathway (Ng et al., 1996). Thus, SRP may block the association of other cytosolic proteins with the polypeptide chain, particularly as long as it is still bound to the ribosome. In addition, it is possible that there may be cytosolic proteins that, although not essential for translocation, specifically recognize the signal sequence and at some point replace SRP. Regardless of whether the cytosolic interaction partners are the same for polypeptides with and without signal sequences, there is the specific issue of how cytosolic proteins are released during posttranslational protein translocation.

Here, we have used a systematic photo-cross-linking approach to probe interactions of cytosolic proteins with translocation substrates during early steps of their posttranslational translocation in yeast, until their binding to the Sec complex. Our results indicate that a posttranslational substrate synthesized in the reticulocyte lysate system interacts with SRP and NAC as long as it is associated with the ribosome. After termination of translation, it interacts with several cytosolic chaperones, including Hsp70 and TRiC/CCT, and has thus likely the same interaction partners as a polypeptide lacking a signal sequence. In fact, no specific cytosolic receptor for signal sequences of posttranslational substrates could be detected. Release of the cytosolic proteins from the translocation substrate occurs spontaneously, and the Sec complex plays no active role. Once bound to the Sec complex, the polypeptide chain is not associated with any cytosolic protein, explaining how its subsequent translocation through the membrane channel can occur without interference of cytosolic proteins.

Materials and Methods

Constructs

cDNA coding for wild-type (wt) ppαF was cloned into the vector pAlter (Promega). All lysine codons in wt ppαF were altered to arginine codons (76, 84, 96, 103, 117, 124, 138, 145, and 159), and single lysines were introduced using appropriate oligonucleotides (Plath et al., 1998). K5Δ ppαF and M2 ppαF are signal sequence mutants of K5 and wt ppαF, respectively, with a deletion of amino acids 10–14 or a substitution of alanine for proline at position 13. The signal sequence of ppαF (165 amino acids) comprises amino acids 1–22.

Transcription, Translation, and Photo–Cross-linking

mRNAs coding for the different full-length ppαF polypeptides and proOmpA were generated as described (Panzner et al., 1995; Plath et al., 1998). Truncated mRNAs coding for 160 amino acids of the respective ppαF molecules were produced after linearization of the plasmid with NciI by in vitro transcription with T7 RNA polymerase. In vitro translation was carried out in a reticulocyte lysate system for 25 min at 30°C in the presence of [35S]methionine and trifluoromethyl-diazirino-benzoic acid (TDBA)-lysyl tRNA (Plath et al., 1998). Translation was stopped by addition of 2 mM cycloheximide. In the case of full-length proteins, ribosomes were removed by centrifugation for 10 min at 100,000 rpm in a Beckman TLA-100 rotor, and cross-links were induced by irradiation with UV light for 15 min on ice. Ribosome–nascent chain complexes (RNCs) were diluted with 10 volumes of buffer A (50 mM Hepes-KOH, pH 7.5, 150 mM potassium acetate, 2 mM magnesium acetate, 5 mM β-mercaptoethanol), and subsequently isolated by centrifugation through a sucrose cushion (buffer A with 500 mM sucrose) for 1 h at 100,000 rpm in a Beckman TLA-100.3 rotor. Finally, RNCs were resuspended in buffer A containing 250 mM sucrose and irradiated with UV light. Truncated nascent chains of 160 residues were released from the ribosome by addition of 10 mM EDTA and ribosome-depleted reticulocyte lysate.

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[1]*Abbreviations used in this paper:* CCT, chaperonin containing TCP1; IP, immunoprecipitation; NAC, nascent polypeptide–associated complex; p60/Hop, Hsp70/Hsp90 organizing protein; ppαF, prepro-α factor; RNC, ribosome–nascent chain complex; SRP, signal recognition particle; TCP1, tailless complex polypeptide; TRiC, TCP1–ring complex; wt, wild-type.

Immunoprecipitations of Cytosolic Cross-linked Products

For immunoprecipitation (IP) after denaturation, SDS was added to the irradiated sample to a final concentration of 2%. The mixture was incubated for 15 min at 65°C, and the SDS concentration was adjusted to 0.1% by dilution with IP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100) containing 1% BSA. Antibodies to Hsp70 (SPA 815 from StressGen Biotechnologies, or K19 [sc-1059] from Santa Cruz Biotechnology, Inc.), to TCP1α (CTA123 from StressGen Biotechnologies; CTA-191, CTA-122, and CTA-184 gave similar results), to Hsp70/Hsp90 organizing protein (p60/Hop) (SRA1500 from StressGen Biotechnologies), to SRP54, and to the α and β subunits of NAC (Wiedmann et al., 1994) were added for 5 h at 4°C and collected with protein G–Sepharose. The resin was washed three times with IP buffer, and the bound material was eluted with SDS sample buffer. For immunoprecipitations under native conditions, the irradiated samples were diluted 20-fold with BT buffer (20 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 0.1% Triton X-100) containing 1% BSA, the antibodies were added for 5 h at 4°C, then collected with protein G–Sepharose, and the resin was washed three times with BT buffer.

Sucrose Gradient Centrifugation and Translocation Assays

30 μl of the irradiated translation mixture containing full-length ppαF or proOmpA was diluted twofold with buffer A, layered on top of a 540-μl sucrose gradient (0.3–1.0 M sucrose in buffer A), and centrifuged for 6 h at 48,000 rpm in a Beckman SW55 rotor. After centrifugation, 50-μl fractions were collected starting from the top. To analyze the translocation competence of different ppαF complexes, a translation mixture containing wt ppαF without photoreactive probes was separated in a sucrose gradient. The translocation assays with different fractions of the sucrose gradients were performed with reconstituted proteoliposomes containing purified yeast Sec complex in the membrane and recombinant Kar2p and ATP in the lumen, as described (Panzner et al., 1995).

Dissociation of Cytosolic Complexes

After in vitro translation of full-length ppαF and sedimentation of ribosomes, 10 μl of the translation mixture was added to 100 μl of buffer A containing 2 mM ATP, 40 mM creatine phosphate, and 1 mg/ml creatine kinase. Where indicated, 1.5 μmol GroEL trap (D87K; provided by F.U. Hartl and his laboratory, Max-Planck-Institute for Biochemistry, Martinsried, Germany), or reconstituted proteoliposomes containing Sec complex were present. After mixing, 10 μl was immediately removed and placed on dry ice to stop the reaction (0.5-min time point). The remainder of the mixture was incubated at 30°C. 10-μl samples were removed at the indicated time points and immediately frozen on dry ice. At the end, all samples were irradiated with UV light for 15 min on dry ice.

To test whether the dissociation of cytosolic complexes leads to a reduction of translocation competence, in vitro–synthesized full-length ppαF was incubated for 30 min at 30°C or 0°C. Subsequently, half of the sample was irradiated with UV light for 15 min on ice, and the other half was incubated with reconstituted proteoliposomes containing Sec complex for binding of ppαF to Sec complex.

IP of ppαF Bound to Sec Complex

Binding of ppαF to reconstituted proteoliposomes containing Sec complex was done essentially as described (Plath et al., 1998). In brief, 4 μl of ppαF translation mixture was either first irradiated for 15 min on ice and subsequently incubated with 30 μl of reconstituted proteoliposomes for 15 min at 30°C, or first incubated with proteoliposomes and then irradiated. The samples were subsequently solubilized in digitonin, and the Sec complex was immunoprecipitated with antibodies to Sec62p.

Electrophoresis

Analysis of all samples was performed by SDS-PAGE with 7.5–17.5% linear acrylamide gels, followed by autoradiography or analysis with a Fuji PhosphorImager BAS1000.
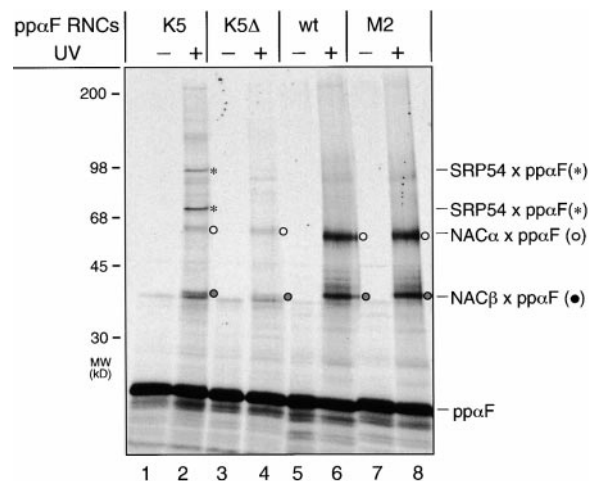

Figure 1. Ribosome-bound ppαF interacts mainly with SRP54 and NAC. Fragments of 160 amino acids of K5, K5Δ, wt, and M2 ppαF containing photoreactive lysine derivatives were synthesized in the reticulocyte lysate system. RNCs were isolated, irradiated with UV light as indicated, and analyzed by SDS-PAGE and autoradiography. Non-cross-linked ppαF and cross-linked products of ppαF to SRP54 (*) and the α (○) and β subunits (●) of NAC are indicated.

Results

Interactions of Ribosome-bound ppαF in the Cytosol

We first used photo-cross-linking to investigate interactions of ribosome-bound nascent ppαF with cytosolic proteins. A truncated mRNA coding for a ppαF polypeptide chain missing only the last five amino acids was translated in vitro in a reticulocyte lysate system in the presence of [^{35}S]methionine. When the ribosome reaches the end of the mRNA, the radioactively labeled nascent chain remains bound to the ribosome as peptidyl tRNA because there is no stop codon to effect termination of translation. The translation system also contained a modified lysyl tRNA that carries a carbene-generating probe in the side chain of the amino acid (Plath et al., 1998). This results in the incorporation of photoreactive lysine derivatives at positions of the polypeptide chain where lysines would normally occur. After translation, RNCs together with bound cytosolic proteins originating from the reticulocyte lysate were isolated by sedimentation, and the sample was irradiated to induce cross-links of nascent ppαF. The cross-linked products were analyzed by SDS-PAGE and autoradiography. With wt ppαF, containing all of its nine lysines in the COOH-terminal half of the polypeptide chain, cross-links occurred mostly to the two subunits of NAC (Fig. 1, lane 6, circles), as demonstrated by IP with antibodies to both NAC subunits after SDS denaturation (data not shown). To identify interaction partners of the signal sequence, we substituted all the lysines of wt ppαF with arginines and introduced a single lysine residue at position 5 within the signal sequence. As previously observed with cotranslational translocation substrates (Wiedmann et al., 1987), the signal sequence could be cross-linked to SRP54 (Fig. 1, lane 2, asterisks). Surprisingly, two bands of SRP54 cross-links were seen, which both could be immunoprecipitated with specific antibodies (data not shown; see also
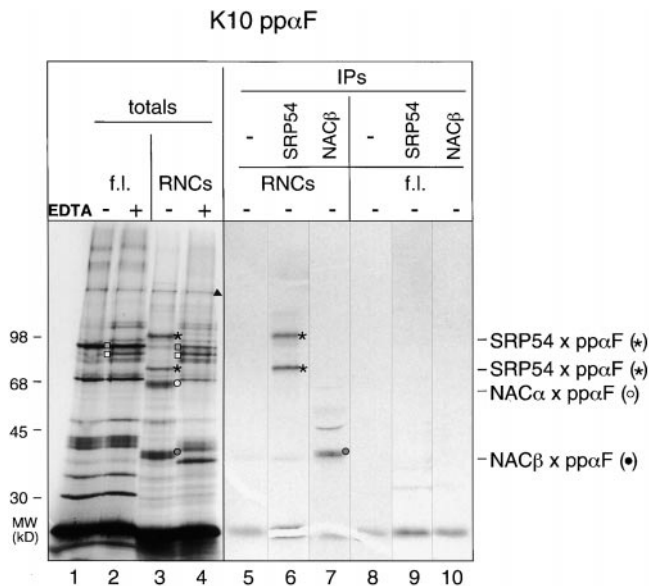
Figure 2. SRP and NAC only interact with RNCs. A fragment of 160 amino acids of ppαF containing a single photoreactive lysine derivative at position 10 (K10 ppαF) was synthesized in vitro. RNCs were isolated, and after addition of ribosome-depleted reticulocyte lysate were incubated with or without EDTA. Full-length (f.l.) ppαF was synthesized in parallel and incubated with or without EDTA. After irradiation, the total products (totals) and products immunoprecipitated with antibodies to SRP54 or NACβ after denaturation in SDS (IP) were analyzed by SDS-PAGE. Control IPs were done without antibodies (−). The starting material for IPs was 3.5 that for the totals. Cross-linked products of ppαF to SRP54 (*) and to the α (○) and β subunits (●) of NAC are indicated. Cross-linked products of ppαF to Hsp70 (■) and TCP1α (□) are also shown. The band marked with a triangle (▲) is an artefact of the gel.

Fig. 2). Perhaps, ppαF is cross-linked to different regions of SRP54, resulting in cross-linked products with different mobilities in SDS gels, as observed with cross-linking of ppαF to Sec61p (Plath et al., 1998). Cross-links to the NAC subunits could also be detected (Fig. 1, lane 2, circles; see also Fig. 2), although they were weaker than those with the probes in the mature region of the chain. These results demonstrate that nascent ppαF interacts mainly with SRP and NAC. It should be noted that nascent ppαF interacts with SRP even when the chain is almost full length, in contrast to the cotranslational translocation substrate preprolactin, which only interacts with SRP when the nascent chain is short (Wiedmann et al., 1987). Thus, the posttranslational substrate ppαF may have folding characteristics that keep the signal sequence exposed.

Next, we tested whether the cross-linking pattern changes when ppαF carries a defective signal sequence (K5Δ and M2 ppαF mutants). M2 and wt ppαF, which contain the photoreactive probes at the same positions in the COOH-terminal domain, showed identical cross-linking patterns (Fig. 1, compare lane 8 with lane 6). However, the K5Δ mutant, containing a single probe at position 5 of its defective signal sequence, showed drastically reduced SRP54 cross-links but no other changes compared with K5 ppαF, containing an intact signal sequence (Fig. 1, compare lane 4 with lane 2). These data demonstrate that the interaction of ppαF with SRP, in contrast to that with NAC, re-

quires a functional signal sequence, confirming previous results obtained with preprolactin (Wiedmann et al., 1994).

Release of SRP and NAC upon Termination of Translation

Next, we studied whether the interaction of ppαF with cytosolic proteins changes upon termination of translation. To generate ribosome-released full-length ppαF, in vitro translation in a reticulocyte lysate system was performed with mRNA containing the natural stop codon, and the ribosomes were removed by centrifugation. In these experiments, we employed a ppαF mutant that carries a single photoreactive probe at position 10 of the signal sequence (K10 ppαF). Ribosome-associated, truncated K10 ppαF of 160 residues gave the same cross-linking pattern as nascent K5 ppαF studied before (Fig. 2, lane 3; compare with Fig. 1, lane 2); both SRP and NAC cross-links could be verified by IP with specific antibodies (Fig. 2, lanes 6 and 7). In contrast, ribosome-released full-length K10 ppαF of 165 residues could not be cross-linked to either SRP or NAC (Fig. 2, lane 1; IPs in lanes 9 and 10), and several new cross-linked products appeared instead (Fig. 2, lane 1). Similar results were obtained when the truncated nascent chain of 160 residues was released from the ribosome with EDTA (Fig. 2, compare lane 4 with lane 3). The resulting cross-linking pattern was similar to that of full-length K10 ppαF (Fig. 2, compare lane 4 with lane 2). These results demonstrate that both SRP and NAC are released from the translocation substrate ppαF upon termination of translation.

Interactions of Full-Length ppαF in the Cytosol

To analyze interactions of full-length ppαF with cytosolic proteins in more detail, we generated 37 ppαF mutants that each contain a single lysine within either the signal sequence or the mature part of the protein. These mutants can be used to scan the environment of distinct regions of the translocation substrate in a systematic manner. All mutant proteins containing photoreactive probes were efficiently bound to the Sec complex and translocated across yeast ER membranes (data not shown; see also Plath et al., 1998).

Each of the single lysine mutants gave cross-links to several cytosolic proteins present in reticulocyte lysate (Fig. 3). In each case, the cross-links were dependent on the presence of photoreactive probes in the polypeptide chain (data not shown). The cross-linking pattern was remarkably similar for all positions probed throughout the polypeptide chain. The major cross-linking partners had molecular masses of ∼200, 180, 70, 62, 60, 50, and 20 kD (in each case, the molecular mass of ppαF [20 kD] was subtracted from the size of the cross-linked product). Some differences between regions were noted. Cross-links to the 70- and 50-kD proteins were more prominent with photoreactive probes at positions within the central part of the signal sequence (positions 5–15) than with probes at all following positions. At position 40, the cross-link to the 200-kD protein disappeared while the band containing the 180-kD protein became more prominent. Beyond position 95, a strong cross-link to a protein of ∼55 kD was observed. The fact that a single position of ppαF could be cross-linked to several proteins suggests that there are different populations of ppαF molecules that contact either different sets of proteins or the same set with different orientations.
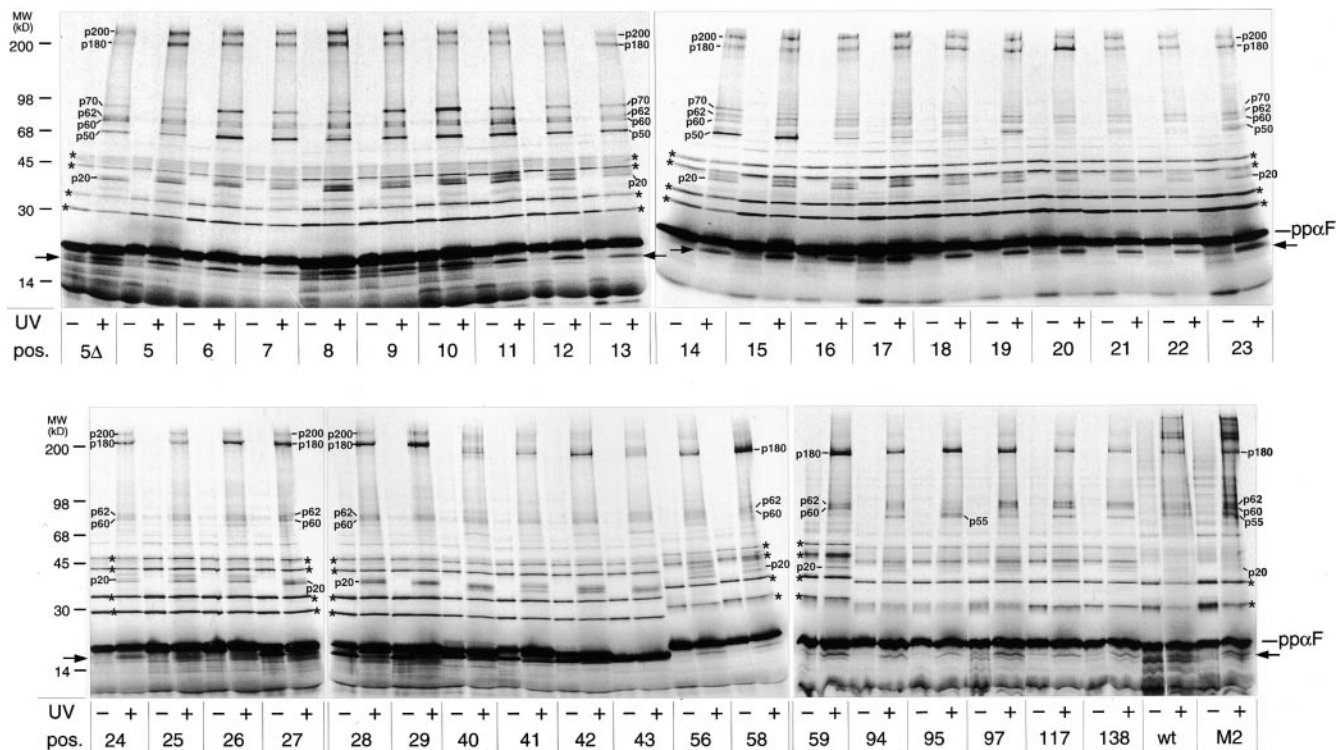
Figure 3. Systematic probing of the molecular environment of full-length ppαF. Full-length polypeptide chains of wt ppαF, of ppαF molecules that each contain a single lysine at the indicated positions (pos.), and of the signal sequence mutants M2 ppαF and K5Δ ppαF were synthesized in vitro in the presence of modified lysyl tRNA. The samples were irradiated with UV light (UV) as indicated and subjected to SDS-PAGE and autoradiography. The position of non-cross-linked ppαF is indicated. Major cross-linked products are indicated by the approximate molecular weight of the cross-linked cytosolic protein. The arrow points to a product originating from internal cross-linking within ppαF. Most bands that are not dependent on irradiation correspond to ubiquitinated ppαF (*) (data not shown). Note that non-cross-linked ppαF runs somewhat differently depending on the position of the lysine codon (in particular, positions 27–43).

With wt ppαF, which contains nine lysine residues in the COOH-terminal half, a complex cross-linking pattern was seen. Several cross-linked products corresponded to those seen with single-lysine mutants (e.g., the cross-links to proteins of ~180, 62, 60, 55, and 20 kD). The many bands seen without irradiation are caused by extensive ubiquitination of ppαF at the lysine residues (data not shown). Taken together, these results show that both the signal sequence and the mature part of newly synthesized full-length ppαF interact with several proteins of the reticulocyte lysate, and that there are relatively small differences in the interaction patterns probed at various positions of the polypeptide chain.

Next, we tested whether the cross-linking pattern changes when ppαF carries a defective signal sequence. Regardless of whether the photoreactive probes were located in the signal sequence (K5Δ ppαF) or in the COOH-terminal region of the polypeptide chain (M2 ppαF), the cross-linking pattern of the signal sequence mutant was indistinguishable from that of the corresponding protein with a functional signal sequence (Fig. 3, compare K5Δ with K5 ppαF, and M2 with wt ppαF; first and last four lanes). These data suggest that there is no cytosolic protein that specifically interacts with the signal sequence of the post-translational translocation substrate. In addition, they suggest that completed polypeptides with and without signal sequence interact with the same set of cytosolic proteins.

We also observed a cross-linked product that migrates in SDS gels slightly faster than ppαF itself (Fig. 3, arrow).

This product is probably generated by internal cross-linking within the ppαF molecule, resulting in a more compact structure with higher mobility in SDS gels. Internal cross-links occurred with some variation in intensity throughout the entire polypeptide chain, suggesting that ppαF may be in a collapsed conformation.

Full-Length ppαF Interacts with Cytosolic Chaperones

To identify the cytosolic cross-linking partners of full-length ppαF, we performed IP experiments with antibodies directed against cytosolic chaperones and their cofactors. First, cross-linked products obtained with ppαF containing the photoreactive probe at position 10 of the signal sequence were analyzed (Fig. 4 A). After denaturation of the irradiated sample in SDS, antibodies to Hsp70 and TCP1α, a subunit of TRiC/CCT, immunoprecipitated cross-linked products of the expected size (Fig. 4 A, lanes 4 and 5, p70 and p62, respectively). Minor cross-links to p60/Hop, a chaperone cofactor that is known to interact with TRiC/CCT, Hsp70, and Hsp90 (Gebauer et al., 1998), could also be identified by denaturing IP (data not shown).

To test whether Hsp70 and TCP1α interact with ppαF alone or in conjunction with other proteins, we performed IPs under native conditions. The efficiency of IP was significantly higher than under denaturing conditions. With both Hsp70 and TCP1α antibodies, the cross-linked product of the expected size corresponded to a major band among the total products (Fig. 4 A, lanes 7 [p70] and 8
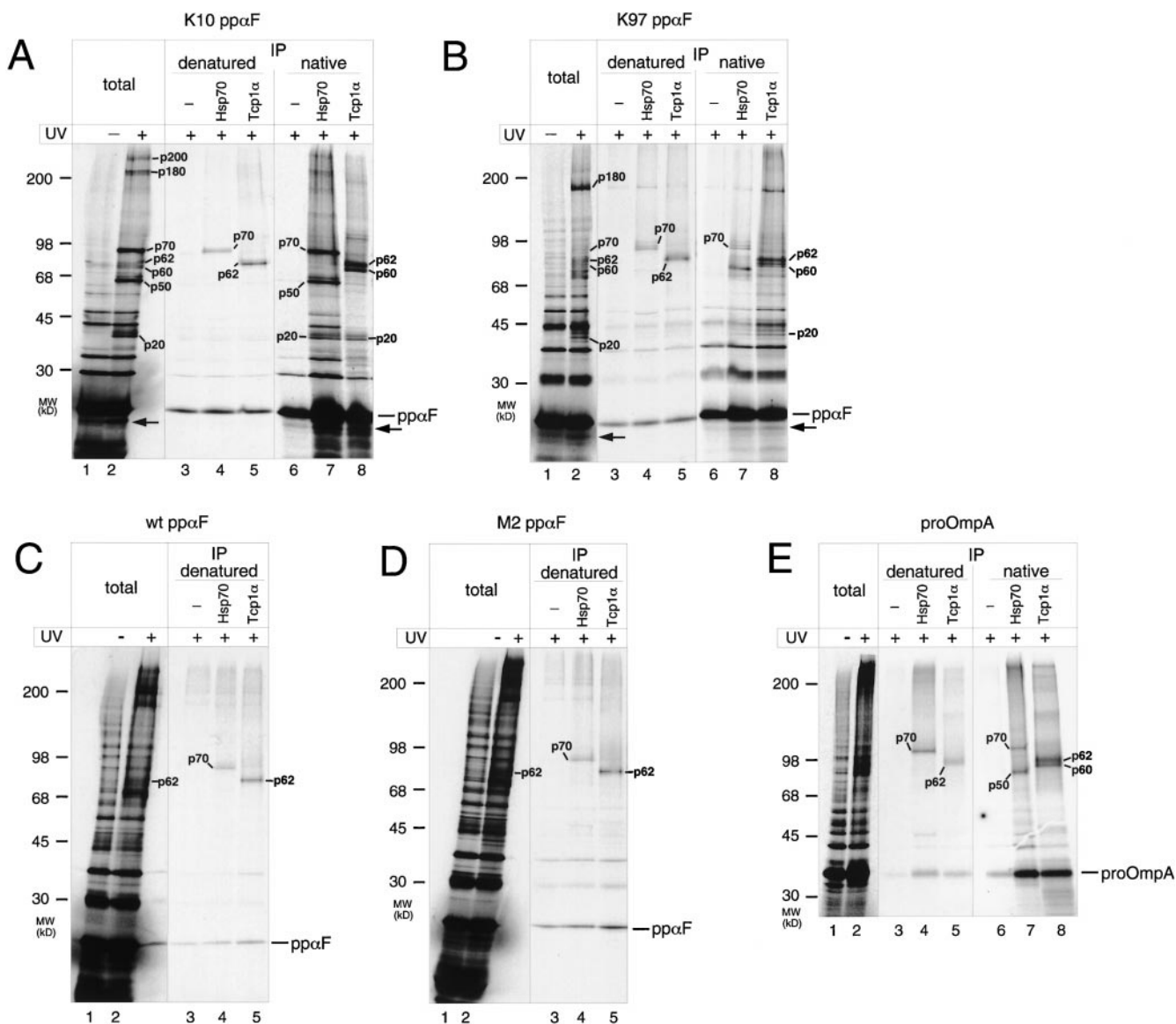
Figure 4. Full-length ppαF interacts with Hsp70 and TCP1α. (A) ppαF containing a photoreactive lysine derivative at position 10 of the signal sequence (K10 ppαF) was synthesized in vitro and irradiated with UV light (UV) as indicated. The samples were either analyzed directly by SDS-PAGE and autoradiography (total), or first immunoprecipitated (IP) with antibodies to Hsp70 and TCP1α before analysis by SDS-PAGE. IPs were performed either under native conditions or after denaturation with SDS. Control IPs were done without antibodies (−). Starting material for the IPs was 10 times that for the totals. Cross-linked products of ppαF are indicated as in the legend to Fig. 3. Note that some non-cross-linked ppαF and ubiquitinated ppαF were coprecipitated in a nonspecific manner, particularly under native conditions. (B) As in A, with ppαF containing a probe at position 97. (C) As in A, with wt ppαF. IPs were performed after denaturation in SDS. (D) As in C, with the signal sequence mutant M2 ppαF. (E) As in A, with proOmpA.

[p62]; compare with lane 2), indicating that both Hsp70 and TCP1α are major interaction partners of the signal sequence of ppαF. Hsp70 antibodies also precipitated the prominent cross-linked products containing the proteins of ∼50 and 20 kD (Fig. 4 A, lane 7). TCP1α antibodies coprecipitated cross-links to a protein slightly smaller than TCP1α itself (p60) as well as products containing the 20-kD protein (Fig. 4 A, lane 8). The native IPs with Hsp70 and TCP1α antibodies together account for the majority of the bands seen among the total cross-linked products (Fig. 4 A, lane 2). These data suggest that ppαF is part of at least two distinct complexes, explaining why different cross-linked bands could be coprecipitated with Hsp70 and TCP1α antibodies under native conditions.

Hsp70 and TCP1α were also identified as cross-linking partners when the photoreactive probes were located in the COOH-terminal region of the ppαF molecule. With the probe at position 97, the pattern of immunoprecipitation with Hsp70 and TCP1α antibodies was very similar to that of ppαF with the probe in the signal sequence, both under denaturing and native conditions (Fig. 4 B). p60/Hop was also identified as a cross-linking partner (data not shown). Similar results were obtained with wt ppαF containing probes in its nine lysine residues at the COOH terminus (Fig. 4 C). As expected from the results described above (Fig. 3), both Hsp70 and TCP1α were also identified as cross-linking partners with M2 ppαF containing a defective signal sequence (Fig. 4, compare D with C;
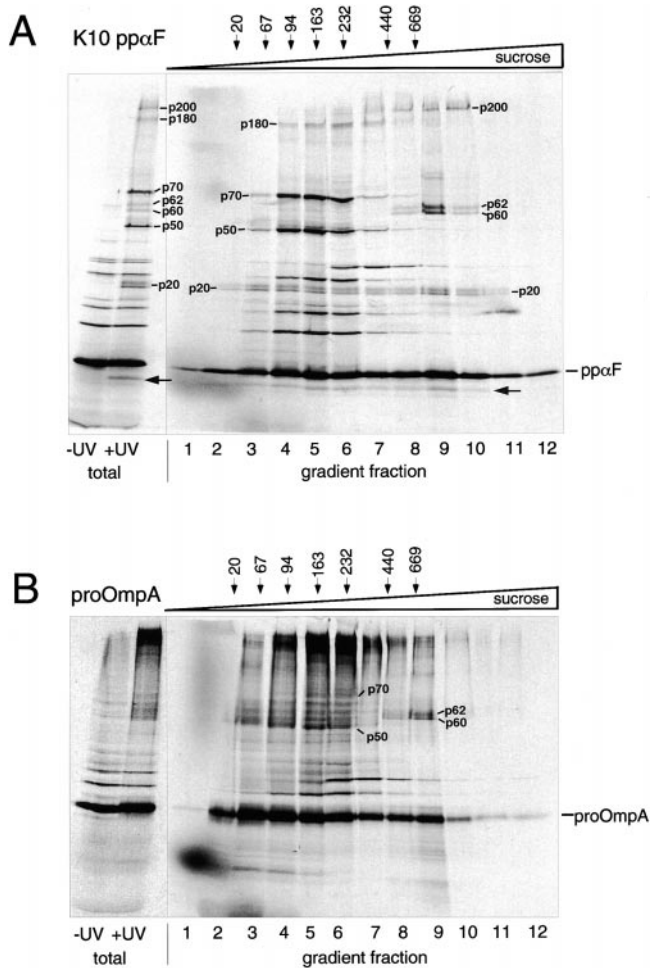
Figure 5. Hsp70 and TRiC/CCT form different complexes with ppαF. (A) K10 ppαF was synthesized in vitro and irradiated with UV light (UV) as indicated (total). An aliquot of the irradiated sample was subjected to sucrose gradient centrifugation, and fractions were collected from the top (lane 1) to the bottom (lane 12). Cross-linked products of ppαF are indicated as in the legend to Fig. 3. Molecular markers were run in parallel sucrose gradients, and their molecular weights are indicated. (B) As in A, with proOmpA.

see also Fig. 3, compare K5Δ with K5 ppαF, p70 and p62). The fact that Hsp70 cross-links more strongly to the signal sequence than to the mature part of ppαF may be explained by its preference for hydrophobic sequences.

Significant cross-links to TCP1α were only observed with ppαF released from the ribosomes (Fig. 2, compare lane 3 with lane 1, □; IP not shown), similar to results obtained with GroEL, the bacterial homologue of TRiC/CCT (Netzer and Hartl, 1998). The results are also in agreement with data obtained with a heterogeneous mixture of nascent chains (Eggers et al., 1997) and with recent data obtained for the cotranslational translocation substrate preprolactin (McCallum et al., 2000). For Hsp70, cross-linking of RNCs was observed, but the intensity of the cross-links became stronger upon termination of translation (Fig. 2, compare lane 3 with lane 1, ■; IP not shown).

We also tested a second posttranslational translocation substrate, proOmpA. Wt proOmpA contains lysine residues both in the signal sequence and in the mature part (a total of 19 lysines). Like ppαF, it could be cross-linked to

several cytosolic proteins in the reticulocyte lysate, although a high background was seen in the absence of irradiation, probably caused by ubiquitination (Fig. 4 E, lanes 1 and 2). IP experiments after denaturation of cross-linked products with SDS demonstrated that proOmpA was cross-linked to Hsp70 and TCP1α (Fig. 4 E, lanes 4 [p70] and 5 [p62]). Native IP suggested again that the translocation substrate is contained in at least two distinct complexes, one with Hsp70 and the other with TCP1α (Fig. 4 E, lanes 7 and 8).

Distinct Translocation-competent Complexes

To confirm that posttranslational translocation substrates are present in at least two distinct complexes with cytosolic proteins, we performed sucrose gradient centrifugation. Specifically, a ppαF mutant containing a single photoreactive probe at position 10 was synthesized in vitro, irradiated, and separated in a sucrose gradient. The cross-links to Hsp70 and the 50-kD protein were found in fractions of relatively low molecular weight (Fig. 5 A, fractions 4–6; IPs for Hsp70 not shown). In fact, the pattern of cross-links in these fractions was remarkably similar to that seen in native IPs with Hsp70 antibodies (Fig. 4 A, lane 7). The cross-links to TCP1α and to the slightly smaller 60-kD protein were found in a high molecular weight peak (Fig. 5 A, fractions 8–10), whose size is consistent with that of TRiC/CCT (970 kD). Again, the cross-linking pattern looked similar to that of native IPs with TCP1α antibodies (Fig. 4 A, lane 8). p60 is thus likely a subunit of TRiC/CCT that contains several polypeptides of almost the same size. The Hsp70 and TRiC/CCT peaks also contained the maximum amounts of non-cross-linked ppαF, although the latter was found in all fractions (quantitation not shown). These data support the existence of at least two distinct ppαF populations, one in a complex with Hsp70 and the other with TRiC/CCT. The other cross-linked products were also found in distinct fractions of the sucrose gradient, and only the cross-links to the 20-kD protein were distributed throughout the gradient. It should be noted that internal cross-links of ppαF were observed in all fractions of the sucrose gradient (Figs. 5 A, arrow), indicating that despite association with different chaperone proteins, ppαF does not attain a fully extended conformation.

Similar results were obtained with wt ppαF and ppaF carrying a photoreactive probe at position 97 of the mature region, with a signal sequence mutant, or if the order of sucrose gradient centrifugation and cross-linking was changed (data not shown). Moreover, proOmpA behaved similarly to ppαF in sucrose gradient centrifugation (Fig. 5 B), indicating that posttranslational translocation substrates are generally associated with different cytosolic chaperone complexes. ppαF synthesized in yeast lysate was also found to be contained in different complexes, since it showed a broad distribution in sucrose gradients (data not shown). Unfortunately, cross-linking experiments with ppαF synthesized in yeast lysates did not give distinct cross-linked bands, most likely because of the fast hydrolysis of the modified lysyl tRNA (data not shown).

Next, we tested whether non–cross-linked ppαF present in the different complexes could be translocated. Wt ppαF without photoreactive probes was separated in a sucrose gradient, and the various fractions of the gradient were in-
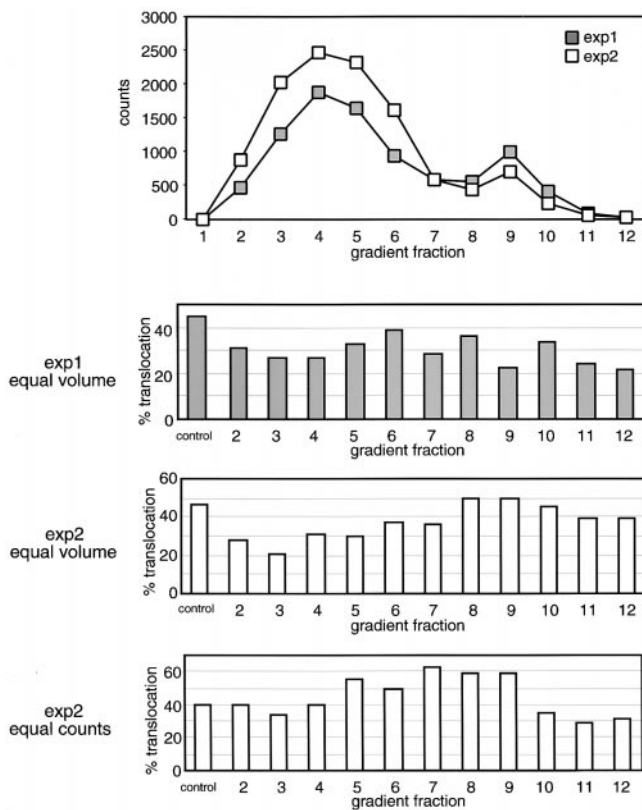
Figure 6. All ppαF populations are translocation competent. Wt ppαF without photoreactive lysine derivatives was synthesized in vitro and subjected to sucrose gradient centrifugation. The top panel shows the distribution of radiolabeled ppαF in the different fractions in two experiments (exp1 and exp2). Translocation of ppαF in the various fractions and in the starting material (control) was tested with proteoliposomes containing Sec complex, Kar2p, and ATP. Either equal volumes or equal amounts of radioactive ppαF were analyzed. % transport, the amount of ppαF translocated relative to the total amount of ppαF in the reaction. Translocation in the absence of proteoliposomes was not observed (data not shown). The increasing amounts of sucrose in the gradient fractions had no influence on the translocation efficiency of ppαF (data not shown).

cubated with proteoliposomes containing Sec complex in the membrane and Kar2p and ATP in the lumen (Fig. 6). Translocation was assessed after treatment with protease. Regardless of whether equal volumes or equal amounts of substrate of the individual fractions were tested, all were clearly active in translocation (Fig. 6). The fact that translocation competence was found for fractions between the TRiC and Hsp70 peaks may be explained either by significant overlap of the peaks or by the presence of a chaperone in all fractions (e.g., p20; see Fig. 5 A). Taken together, these results indicate that different sets of cytosolic proteins keep ppαF in a translocation-competent conformation.

Dissociation of ppαF from Cytosolic Proteins

We next investigated the fate of the ppαF–chaperone complexes during the next step in posttranslational protein translocation, the binding of ppαF to the Sec complex. Specifically, ppαF containing a photoreactive probe at a single position was synthesized in vitro in a reticulocyte lysate system, the ribosomes were removed by centrifuga-

tion, and proteoliposomes containing the purified yeast Sec complex were added. The vesicles lack Kar2p and thus allow binding of ppαF to the cytosolic face of the Sec complex but no translocation (Plath et al., 1998). After different incubation times at 30°C, the samples were irradiated and the cross-linked products were separated by SDS-PAGE and quantitated. For these experiments, we used ppαF with a photoreactive probe at position 11 because it gives only weak cross-links to the Sec61p subunit of the Sec complex (see Fig. 8 A, lane 6), which could obscure the presence of some cross-links to cytosolic proteins in SDS gels. When the Sec complex was present, the cross-links to the 50-kD protein and to Hsp70 both diminished very rapidly with the same kinetics (Fig. 7, A and B). Cross-links to the p60 subunit of TRiC/CCT also decreased, but more slowly (Fig. 7 C). The kinetics of dissociation of cytosolic proteins from ppαF are consistent with those of binding of ppαF to the Sec complex, as determined by the appearance of cross-links to Sec62p and Sec71p, two other subunits of the Sec complex (Fig. 7 D). The cross-links to the proteins of ∼20 kD also disappeared rapidly (those to the 180- and 200-kD proteins could not be well quantitated; data not shown).

When the proteoliposomes were omitted, cross-links to Hsp70, p60, and the 50-kD protein also diminished with time but remained significantly stronger than in the presence of Sec complex throughout the experiment (Fig. 7, A–C, control; cross-links to the 20-kD proteins behaved similarly [data not shown]). Liposomes lacking Sec complex gave the same result (data not shown). Incubation of ppαF–chaperone complexes on ice resulted in all cross-links remaining constant (data not shown; see also Fig. 7 E). These results show that, at elevated temperatures, spontaneous net dissociation of complexes between ppαF and cytosolic proteins occurs; net dissociation is significantly faster in the presence of Sec complex.

To test whether the Sec complex stimulates dissociation in an active manner or simply captures free ppαF molecules spontaneously released from their cytosolic partners, we used a mutant of the *Escherichia coli* chaperonin GroEL as a passive trap (Fenton et al., 1994). This mutant (D87K, in which the aspartate at position 87 is replaced by a lysine) is able to bind unfolded proteins, but cannot release them and is not expected to actively stimulate the dissociation of ppαF from cytosolic proteins (Thulasiraman et al., 1999). In the presence of the GroEL trap, the cross-links to all cytosolic proteins disappeared with similar rapid kinetics as in the presence of the Sec complex (Fig. 7, A–C). In addition, cross-links to GroEL appeared with the same kinetics as those to proteins of the Sec complex (Fig. 7 D). We conclude that in both cases the binding partner serves as a simple trap for spontaneously released ppαF molecules. Dissociation of the complexes between ppαF and cytosolic proteins seems to be the rate-limiting step in the transfer of the substrate to the respective binding partner.

To test whether the spontaneous dissociation of chaperone–substrate complexes in the absence of Sec complex leads to a reduction of translocation competence, we preincubated in vitro–synthesized ppαF at 30°C or 0°C, and then tested in parallel cross-linking of ppαF to cytosolic proteins and binding of ppαF to the Sec complex (Fig. 7 E). While after preincubation on ice both the cross-linking to cytosolic chaperones and the binding to the Sec com-
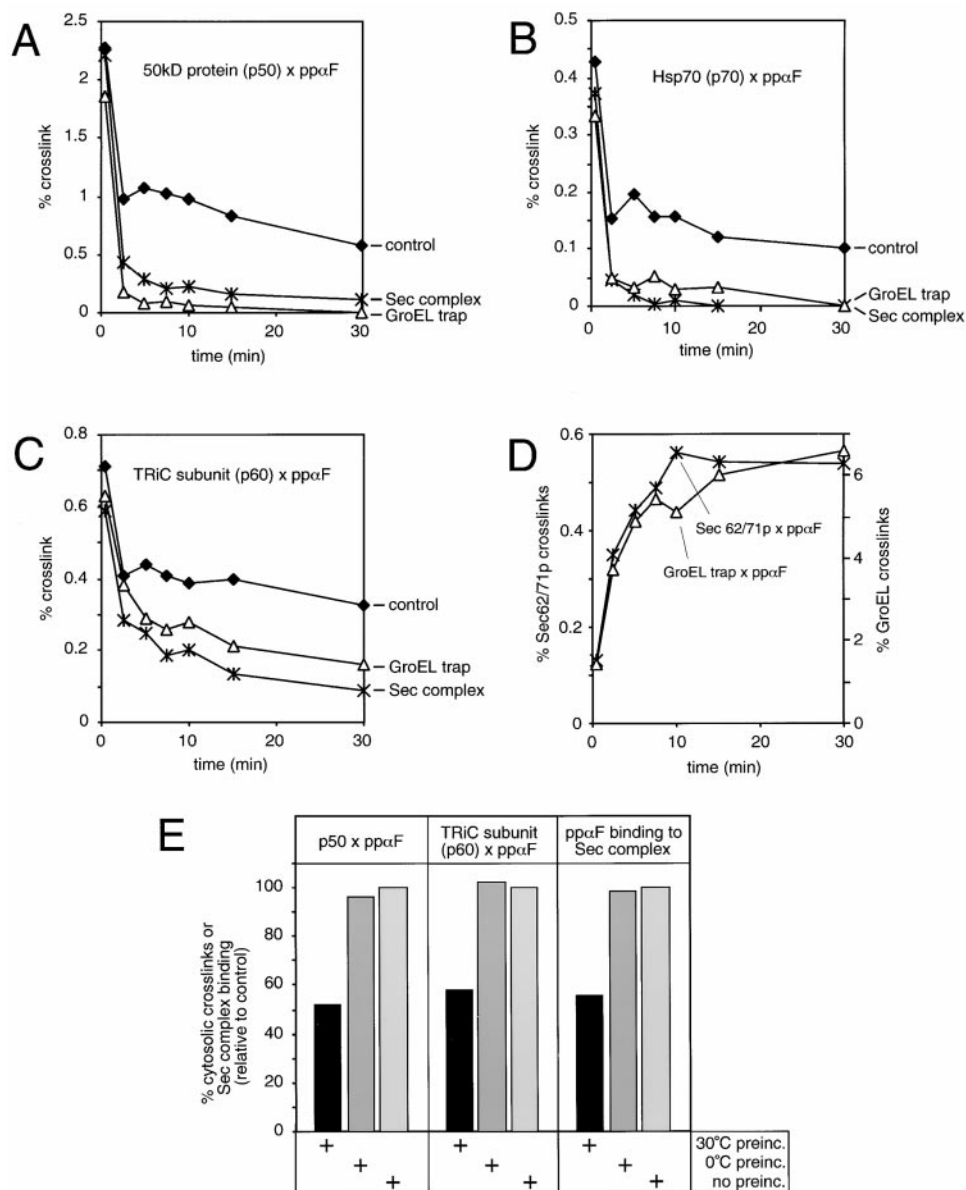
Figure 7. Dissociation of cytosolic complexes of ppαF. (A) K11 ppαF was synthesized in reticulocyte lysate and incubated at 30°C either without additions (◆, control), or with a GroEL trap (△), or with proteoliposomes containing Sec complex (×). At different time points, equal aliquots were removed from the samples, irradiated with UV light, and analyzed by SDS-PAGE. The cross-linked product containing the 50-kD protein (p50) was quantitated with a PhosphorImager. The yield of cross-linked product is expressed relative to the amount of ppαF contained in the sample. The first time point was taken after 0.5 min, which already led to some binding of ppαF to the Sec complex or GroEL (see D) and to less cross-linking to cytosolic proteins compared with the control. (B) As in A for Hsp70 (p70). (C) As in A for the p60 subunit of the TRiC complex. (D) Quantitation of the ppαF cross-links to Sec62 and Sec71p (for the sample incubated with Sec complex) and to GroEL (for the sample incubated with the GroEL trap). (E) K11 ppαF was synthesized in reticulocyte lysate and incubated for 30 min at 30°C or 0°C without additions as indicated (preinc.). Half of the sample was irradiated with UV light and analyzed by SDS-PAGE. The cross-linked products containing the 50-kD protein (p50) and the TRiC subunit p60 were quantitated with a PhosphorImager. The other half of the sample was analyzed for binding of ppαF to Sec complex. The sample was incubated with proteoliposomes containing Sec complex, solubilized in digitonin, and subjected to IP for the Sec complex. ppαF coimmunoprecipitated was quantitated. The yield of cross-linked products and the efficiency of Sec complex binding are given relative to results obtained with a sample that was immediately subjected to cross-linking and binding without preincubation (control = 100%).

plex remained unchanged compared with a sample without preincubation, at 30°C both were reduced to the same extent (~50%). These data suggest that substrate molecules released from cytosolic chaperones aggregate and become incompetent for translocation if they cannot immediately interact with the Sec complex.

Dissociation of Cytosolic Complexes Required for ppαF–Sec Complex Interaction

To confirm the release of cytosolic proteins from ppαF during initiation of translocation, we analyzed interactions of the substrate bound to the Sec complex. ppαF molecules with probes in the signal sequence at positions 11 or 13 were first incubated with proteoliposomes containing the purified Sec complex and then irradiated (Fig. 8, A and B, 1.B/2.X). The samples were either analyzed directly

by SDS-PAGE (total) or solubilized in digitonin, and were then subjected to IP with antibodies to Sec62p to isolate the Sec complex and the associated cross-linked and non–cross-linked ppαF. As reported previously (Plath et al., 1998), ppαF with a probe at position 11 cross-linked weakly to Sec61p and strongly to Sec62p and Sec71p (the latter are not separated in the gel; Fig. 8 A, lane 6). ppαF with a probe at position 13 gave strong cross-links to both Sec61p and Sec62p/71p (Fig. 8 B, lane 6). With both positions of the probe, ppαF bound to Sec complex did not give any cross-link to cytosolic proteins (Fig. 8, A and B, lane 6), indicating that the cytosolic binding partners were released from the signal sequence upon its binding to the Sec complex. To test whether cytosolic proteins are also released from the mature portion of ppαF, we repeated the experiments with mutants containing probes at positions 117, 138, and with the wild-type protein containing
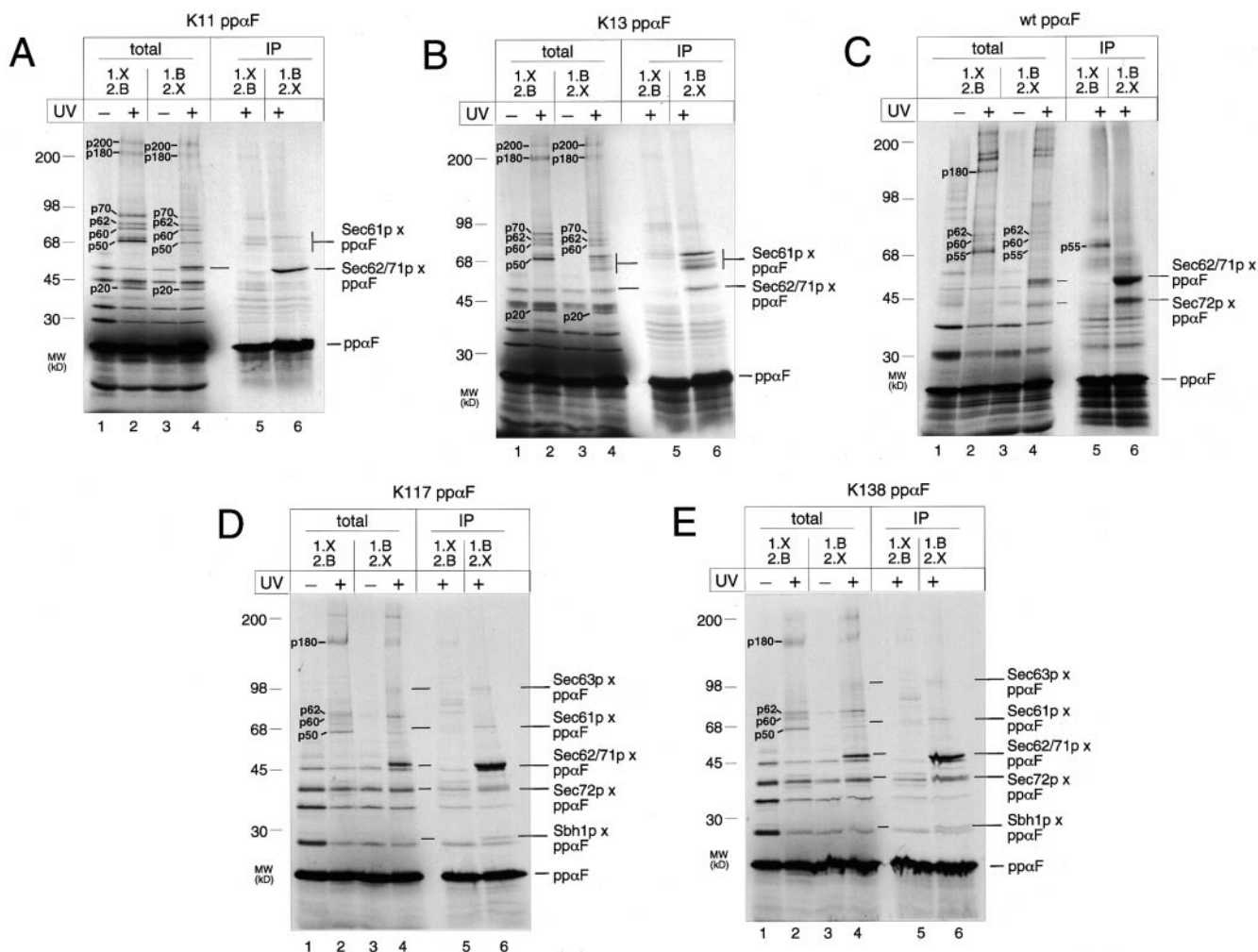
Figure 8. ppαF bound to Sec complex is not associated with cytosolic proteins. (A) K11 ppαF was synthesized in vitro. Some samples were first irradiated with UV light and then incubated with reconstituted proteoliposomes containing Sec complex (1.X/2.B). Other samples were first incubated with proteoliposomes and then irradiated with UV light (1.B/2.X). One part of each sample was analyzed directly by SDS-PAGE and autoradiography (total). Seven parts of the irradiated samples were treated with digitonin to solubilize the vesicles, and ppαF bound or cross-linked to components of the Sec complex was coimmunoprecipitated with the Sec complex (IP). Cytosolic interaction partners of ppαF are indicated as in the legend to Fig. 3. Cross-links to the Sec proteins are marked. (B) As in A, with K13 ppαF. (C) As in A, with wt ppαF. (D) As in A, with K117 ppαF. (E) As in A, with K138 ppαF.

the probes at nine COOH-terminal positions (Fig. 8, C–E). With all three proteins, strong cross-links to Sec62/71p and Sec72p were seen, while the single-lysine mutants showed additional very weak cross-links to Sec63p, Sec61p, and Sbh1p (Sec72p, Sec63p, and Sbh1p are also subunits of the Sec complex). Significantly, in all cases no cytosolic cross-links of ppαF bound to the Sec complex were discernible (Fig. 8, C–E, lane 6). Thus, during the binding of ppαF to the Sec complex, all cytosolic proteins must have been released, even from COOH-terminal parts of the polypeptide chain which are not inserted into the translocation channel.

Finally, we tested whether the release of cytosolic proteins is required for the binding of ppαF to the Sec complex. To this end, samples containing ppαF with photoreactive probes at different positions were first irradiated on ice, conditions that maximize the extent of cross-linking to cytosolic proteins, and then proteoliposomes containing the Sec complex were added to allow binding of ppαF to the Sec complex (Fig. 8, A–E, 1.X/2.B). Again, both the total

products and those associated with the Sec complex were analyzed. Although a large number of cross-links to cytosolic proteins were visible among the total products, most of them either were not bound or were only inefficiently bound to the Sec complex (Fig. 8, A–E, compare lanes 2 and 5). The only clear exception are the cross-links to the 55-kD protein (p55), seen with probes in wt ppαF, which were coprecipitated with the Sec complex. Thus, cross-linking of most cytosolic proteins appears to prevent the interaction of ppαF with the Sec complex, suggesting that their release is a prerequisite for initiation of translocation.

Discussion

We have used photo-cross-linking to follow the route of a posttranslational translocation substrate from its synthesis on a free ribosome to its binding to the Sec complex, the component in the yeast ER membrane that forms the translocation channel. Our data suggest the following sequence of events (Fig. 9). First, while still bound to the ri-
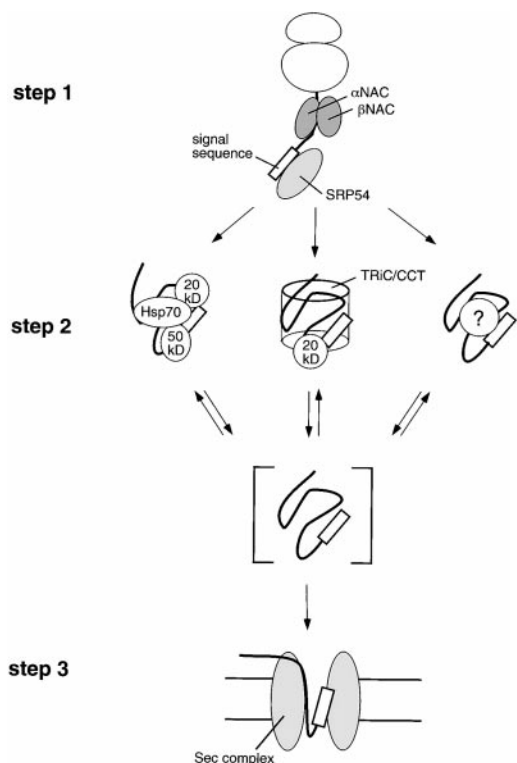
Figure 9. Pathway of a posttranslational translocation substrate from the ribosome to the Sec complex. For explanation, see text.

bosome, the nascent polypeptide chain interacts with SRP and NAC. Second, when the chain is completed and released from the ribosome, it no longer interacts with these proteins and instead associates with several different cytosolic proteins. At least two distinct complexes could be identified, one containing Hsp70 and the other TRiC/CCT. Third, the cytosolic complexes dissociate spontaneously, without active participation of the Sec complex. Finally, substrate bound to the Sec complex is no longer associated with any cytosolic proteins and can now begin translocation through the membrane channel without interference by cytosolic proteins.

For technical reasons, we have used in our experiments a heterologous system comprised of cytosol from rabbit reticulocytes and Sec complex from *S. cerevisiae*. However, we believe that the results are also relevant to the homologous system in yeast, and even to in vivo conditions, for the following reasons. First, ppαF and proOmpA synthesized in a reticulocyte lysate system are posttranslationally transported into yeast ER vesicles with the same high efficiency as substrates synthesized in yeast lysate (data not shown). Although posttranslational translocation appears to be more prominent with yeast ER membranes than with mammalian ones, the difference is not due to cytosolic factors. Second, the cytosolic chaperone proteins are highly conserved among species, making it unlikely that entirely different pathways are employed in different eukaryotes. Furthermore, cytosolic chaperone proteins are equally abundant both in reticulocyte and yeast lysates (Frydman et al., 1994). Third, in sucrose gradients, ppαF synthesized in yeast lysate runs as a heterogeneous population, similarly to ppαF made in reticulocyte lysate. Fourth, in yeast, Hsp70 is involved in the translocation of

ppαF both in vivo and in vitro (Chirico et al., 1988; Deshaies et al., 1988; Becker et al., 1996), similarly to its role in our heterologous system. Despite these arguments, we cannot strictly exclude that yeast chaperones, in contrast to mammalian chaperones, are actively dissociated from the substrate by the Sec complex, but the data demonstrate that active dissociation is not required for translocation and, if it occurred, would have little effect on the translocation efficiency.

Our results show that a posttranslational translocation substrate differs from a polypeptide lacking a signal sequence, as long as it is associated with the ribosome, by being associated with SRP. However, once released from the ribosome, both classes of proteins seem to interact with the same cytosolic proteins. These include Hsp70 and TRiC/CCT, but also p60/Hop. Several other, unidentified binding partners of posttranslational translocation substrates may also be identical to those known to interact with cytosolic polypeptides. The 50-kD protein might be Hsp40, a mammalian cytosolic J protein, since it comigrated with Hsp70 in sucrose gradients and could be coimmunoprecipitated with it. The cross-linked proteins of ~20 kD might be subunits of prefoldin, a cofactor of TRiC/CCT that has been shown to interact with some newly synthesized polypeptides (Hansen et al., 1999). Unfortunately, no cross-links could be precipitated with Hsp40 or prefoldin antibodies (data not shown). No evidence was seen for cytosolic factors that interact specifically with the signal sequence of completed chains, suggesting that the signal sequence of a posttranslational substrate is only recognized by the Sec complex and not by a cytosolic signal sequence receptor. Thus, the only function of cytosolic binding partners may be to keep a polypeptide in a loosely folded conformation, a function not specific for translocation substrates. The essential role of cytosolic chaperones in translocation is illustrated by our observation that a decrease in cross-linking to cytosolic proteins after incubation at elevated temperature results in reduced substrate binding to the Sec complex (see Fig. 7 E). Our data also support the idea that there may be more than one way to keep a polypeptide unfolded and thus competent for translocation. Redundant pathways are also suggested by in vivo data in yeast, in which depletion of functional Hsp70 affects the posttranslational translocation of some substrates but not of others (Becker et al., 1996).

Our data suggest that the cytosolic proteins continuously associate with and dissociate from a completed polypeptide chain. The addition of an unspecific GroEL trap accelerated the net dissociation of all chaperones, indicating that in its absence a portion of the substrate is able to reassociate with them. Reassociation of chaperones is apparently not very efficient in our in vitro system. In vivo, the chaperones may associate and dissociate as long as the translocation substrate remains in the cytosol (see also Eggers et al., 1997), since the polypeptide cannot reach its native conformation in this compartment.

Given that completed polypeptides with and without a signal sequence apparently interact with the same cytosolic proteins, and that there is continuous binding and dissociation, it may not be very surprising that we found that the Sec complex plays no active role in releasing these proteins from the translocation substrate. This conclusion is based on the result that the Sec complex and the unspe-

cific GroEL trap accelerate net dissociation to the same extent. The binding of the substrate to these components appears to prevent the reassociation with cytosolic proteins. Surprisingly, Sec complex and the GroEL trap even stimulated the disappearance of all cross-links after depletion of ATP by addition of hexokinase and glucose (data not shown), suggesting that dissociation of the chaperones can occur in their ADP form. A similar observation has been made for the release of cytosolic Hsp70 from mitochondrial precursor proteins (Komiya et al., 1996).

Eventually, the polypeptide chain bound to the Sec complex is entirely stripped of all cytosolic proteins. It is possible that the polypeptide chain needs to be fully stripped before its binding to the Sec complex because, with the exception of a 55-kD protein, all cytosolic proteins need to be released from the substrate to allow its stable interaction with the Sec complex. Alternatively, only the signal sequence may have to be stripped initially, allowing substrate binding to the Sec complex. The chaperones would then dissociate from the mature part of the chain but reassociation would be prevented, possibly by the large cytosolic domains of the Sec62p and Sec63p subunits of the Sec complex.

Remarkably, once bound to the Sec complex, the substrate is free of cytosolic proteins even in regions that are protruding into the cytosol or are only loosely associated with the cytosolic aspect of the Sec complex. As the bound polypeptide chain is fully stripped, it can move freely through the channel, uninhibited by any interaction with cytosolic proteins. In fact, thermal motion has been demonstrated to be sufficient for the movement of a chain through the channel (Matlack et al., 1999). The existence of a "naked" polypeptide chain would allow a Brownian ratcheting mechanism to operate and would also facilitate a mechanism in which Kar2p would pull on the polypeptide chain and accelerate its forward movement through the channel.

Posttranslational translocation appears to be quite different from the tightly regulated process of cotranslational translocation in which the signal sequence is recognized first by SRP in the cytosol and then by the Sec61p complex in the membrane. In the posttranslational pathway, the only essential recognition step seems to be the binding of the signal sequence to the Sec complex. Although we have found that the signal sequence of a ribosome-bound posttranslational substrate can bind to SRP, the interaction may be of low affinity in living yeast cells, explaining why the substrate escapes cotranslational membrane targeting. The recognition of the signal sequence by the Sec complex must be sufficient to distinguish proteins to be exported from those staying in the cytosol, despite the fact that both can associate with the same chaperones. The situation may be similar for at least some proteins imported into mitochondria. Although some targeting sequences may be recognized by specific cytosolic receptors, others associate with Hsp70 and are only recognized by receptors on the outer mitochondrial membrane (Komiya et al., 1996).

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